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A reliable and inexpensive method of nucleic acid extraction for the PCR-based detection of diverse plant pathogens

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A B S T R A C T

A reliable extraction method is described for the preparation of total nucleic acids from at least ten plant genera for subsequent detection of plant pathogens by PCR-based techniques. The method combined a modified CTAB (cetyltrimethylammonium bromide) extraction protocol with a semi-automatic homogenizer (FastPrep® instrument) for rapid sample processing and low potential of cross contamination. The method was applied to sample preparation for PCR-based detection of 28 different RNA and DNA viruses, six viroids, two phytoplasmas and two bacterial pathogens from a range of infected host plants including sweet potato, small fruits and fruit trees. The procedure is cost-effective and the qualities of the nucleic acid preparations are comparable to those prepared by commonly used commercial kits. The efficiency of the procedure permits processing of numerous samples and the use of a single nucleic acid preparation for testing both RNA and DNA genomes by PCR, making this an appealing method for testing multiple pathogens in certification and quarantine programs.

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1. Introduction

Specific amplification of target nucleic acid sequences by polymerase chain reaction (PCR), reverse transcription-PCR (RT-PCR) or real-time PCR techniques is widely used for identification and detection of plant pathogens (Mumford et al., 2006). The PCR-based detection assays are highly specific, sensitive and rapid, and utilize reagents that are easily accessible and relatively inexpensive. For some plant pathogens, PCR assays would provide a significant improvement over other available detection assays such as biological indicators. However, successful application of these methods for pathogen detection requires efficient recovery of target nucleic acids with minimal PCR inhibitors such as polyphenolic and polysaccharide compounds from infected plants. This is especially true for pome, prunus and citrus fruit trees, small fruits, sweet potatoes and some ornamental plants (Demeke and Adams, 1992; Henson and French, 1993; Jones and McGavin, 2002; Korschineck et al., 1991; Nassuth et al., 2000; Staub et al., 1995; Tairo et al., 2006). Different methods have been developed for extraction of nucleic acids from various types of plants for pathogen detection. In many cases, the methods have been described for each pathogen and/or crop individually. Various commercial extraction kits such as RNeasy and DNeasy Plant Mini Kits (Qiagen, Valencia, CA, USA) are available but they may not be suitable for plants rich in polyphenolic and polysaccharide compounds and they can be relatively expensive. Many of these methods are laborious, time-consuming and pose a higher risk of cross contamination between samples due to manual homogenization of plant samples. These factors become very significant in certification and quarantine programs of vegetatively propagated plant materials for which large numbers of samples need to be tested for multiple pathogens.

Several different protocols such as the RNeasy and DNeasy kits had been used to extract nucleic acids from various plants for the PCR-based detection of pathogens with different genomes, i.e. ssRNA, ssDNA and dsDNA in our laboratories. To improve efficiency, it was important to determine if any of the nucleic acid extraction protocols could be modified and utilized as a 'universal' nucleic acid extraction procedure for PCR-based detection of multiple pathogens in a single extract of plant samples, irrespective of genome composition of the pathogens and plant types. CTAB, cetyltrimethylammonium bromide, is a strong ionic denaturing detergent. This surfactant has been used to facilitate the separation of proteins from nucleic acids in extractions of biological materials. The CTAB method was first developed to isolate DNA from barley seedlings by Doyle and Doyle (1987) using the buffer system reported by Saghai-Maroof et al. (1984) and has been widely used to obtain high-quality nucleic acids from plants for various

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Table 1
List of pathogens and PCR results from samples prepared by CTAB method and QIAGEN kits.

Pathogens			Host species	PCR result	
Species	Genus	Family		CTAB	QIAGEN
RNA viruses					
Apple chlorotic leaf spot virus (ACLSV)	*Trichovirus*	*Flexiviridae*	*Malus domestica*	+	nd*
Apple stem grooving virus (ASGV)	*Capillovirus*	*Flexiviridae*	*Malus domestica*	+	+
Apple stem pitting virus (ASPV)	*Foveavirus*	*Flexiviridae*	*Malus sieversii*	+	+
Blackcurrant reversion (BRV)	*Nepovirus*	*Comoviridae*	*Ribes nigrum*	+	–
American plum line pattern virus (APLPV)	*Ilarvirus*	*Bromoviridae*	*Prunus tomentosa*	+	+
Cherry green ring mottle virus (CGRMV)		*Flexiviridae*	*Prunus persica*	+	+
Cherry necrotic rusty mottle virus (CNRMV)		*Flexiviridae*	*Prunus serrulata*	+	+
Cherry virus A (CVA)	*Capillovirus*	*Flexiviridae*	*Prunus mahaleb*	+	+
Little cherry virus 1 (LChV-1)		*Closteroviridae*	*Prunus avium*	+	nd
Plum pox virus (PPV)	*Potyvirus*	*Potyviridae*	*Prunus persica*	+	+
Prune dwarf virus (PDV)	*Ilarvirus*	*Bromoviridae*	*Prunus tomentosa*	+	+
Prunus necrotic ringspot virus (PNRSV)	*Ilarvirus*	*Bromoviridae*	*Prunus tomentosa*	+	+
Barley yellow dwarf virus (BYDV)-MAV	*Luteovirus*	*Luteoviridae*	*Hordeum vulgare*	+	nd
BYDV-PAV	*Luteovirus*	*Luteoviridae*	*Hordeum vulgare*	+	nd
Cereal yellow dwarf virus (CYDV)-RPV	*Luteovirus*	*Luteoviridae*	*Hordeum vulgare*	+	nd
Lolium latent virus (LoLV)		*Flexiviridae*	*Lolium perenne*	+	+
Ryegrass mosaic virus (RGMV)	*Rymovirus*	*Potyviridae*	*Lolium perenne*	+	+
Fiji disease virus (FDV)	*Reovirus*	*Reoviridae*	*Saccharum* spp.	+	+
Sugarcane mosaic virus (SCMV)	*Potyvirus*	*Potyviridae*	*Saccharum* spp.	+	+
Sorghum mosaic virus (SCMV)	*Potyvirus*	*Potyviridae*	*Saccharum* spp.	+	+
Sugarcane streak mosaic virus (SCMV)		*Potyviridae*	*Saccharum* spp.	+	+
Sugarcane yellow leaf virus (SCYLV)	*Polerovirus*	*Luteoviridae*	*Saccharum* spp.	+	+
Sweet potato feathery mottle virus (SPFMV)	*Potyvirus*	*Potyviridae*	*Ipomoea batatas*	+	+
Sweet potato virus G (SPVG)	*Potyvirus*	*Potyviridae*	*Ipomoea batatas*	+	+
Sweet potato virus Y (SPGY)	*Potyvirus*	*Potyviridae*	*Ipomoea batatas*	+	+
Sweet potato chlorotic stunt virus (SPCSV)	*Crinivirus*	*Closteroviridae*	*Ipomoea batatas*	+	+
Viroids					
Apple dimple fruit viroid (ADFVd)	*Apscaviroid*	*Pospiviroidae*	*Malus domestica*	+	+
Apple fruit crinkle viroid (AFCVd)		*Pospiviroidae*	*Malus domestica*	+	+
Apple scar skin viroid (ASSVd)	*Apscaviroid*	*Pospiviroidae*	*Malus domestica*	+	+
Pear blister canker viroid (PBCVd)	*Apscaviroid*	*Pospiviroidae*	*Malus domestica*	+	+
PBCVd	*Apscaviroid*	*Pospiviroidae*	*Pyrus communis*	+	+
Hop stunt viroid (HSVd)	*Hostuviroid*	*Pospiviroidae*	*Prunus salicina*	+	+
Peach latent mosaic viroid (PLMVd)	*Pelamoviroid*	*Avsunviroidae*	*Prunus persica*	+	+
DNA viruses					
Gooseberry vein banding associated virus (GVBaV)	*Badnavirus*	*Caulimoviridae*	*Ribes rubrum*	+	+
Sweet potato leaf curl virus (SPLCV),	*Begomovirus*	*Geminiviridae*	*Ipomoea batatas*	+	+
Phytoplasmas					
Candidatus Phytoplasma mali	Phytoplasma	Acholeplasmataceae	*Malus domestica*	+	+
Candidatus Phytoplasma pyri	Phytoplasma	Acholeplasmataceae	*Prunus persica*	+	+
Bacteria					
Ralstonia solanacearum K60	*Ralstonia*	Burkholderiaceae	*Pelargonium* × *hortorum*	+	-
Candidatus Liberibacter asiaticus B232	Liberibacter	Rhizobiaceae	*Citrus jambhiri*	+	+

applications (Allen et al., 2006; Bekesiova et al., 1999; Chang et al., 1993; Flagel et al., 2005; Jaakola et al., 2001; Kim and Hamada, 2005; Puchooa, 2004; Wang et al., 2005, 2008). CTAB-based methods have also been used to prepare samples from infected plants for PCR-based detection of DNA viruses, bacteria and phytoplasmas (Francis et al., 2006; Hren et al., 2007; Hu et al., 1996; Li et al., 2006; Wyatt and Brown, 1996), but its application for PCR-based detection of plant RNA viruses and viroids is less reported (Chang et al., 2007; Harjua et al., 2005; López et al., 2006; Mumford et al., 2000; Parmessur et al., 2002). This paper reports the development of a simple, reliable, inexpensive and universal CTAB-based method for extraction of nucleic acids for PCR-based detection of a range of plant pathogens including viruses, viroids, phytoplasmas and bacteria.

2. Materials and methods

2.1. Pathogen sources

Isolates of different viruses, viroids, phytoplasmas and bacteria used in this study are listed in Table 1. The viruses, viroids and phytoplasmas were maintained in either naturally infected plants or graft inoculated host plants in insect-proof greenhouses. Isolate B232 of *Candidatus* Liberibacter asiaticus (Las B232), the causal agent of citrus huanglongbing (HLB, ex greening), was obtained from Thailand, graft inoculated into rough lemon and maintained in a secure greenhouse. Pathogen-free host plants were used as negative controls in all assays.

The wild-type strain K60 of *Ralstonia solanacearum* E. F. Smith race 1 biovar 1 was freshly streaked from a water stock onto a casamino acid peptone glucose (CPG) medium plate, then a single colony was grown in CPG broth overnight (Kelman, 1954), and used to inoculate geranium (*Pelargonium* × *hortorum*) seedlings 14 days after transplanting by pouring 40 ml of bacterial inoculum into each pot (2×10^7 CFU/ml). Water was used as a negative control. The inoculated plants were maintained in a greenhouse at 30 °C with 14 h of light. The plants were rated using a disease index (DI) scale as follows: 0, no wilted leaves; 1, ≤25% wilted; 2, 26–50% wilted; 3, 51–75% wilted; and 4, 76–100% wilted or dead (Roberts et al., 1988). One week after inoculation, water-inoculated healthy plants (DI = 0) and plants with DI of 1 and 4 were collected for DNA extraction and PCR assay.

2.2. Extraction of nucleic acids

Appropriate plant samples (leaves, buds, stems, petioles and/or bark) were collected from several positions of a test plant and torn by hand (soft tissues) or cut with a razor blade (woody tissues) into small pieces. Pooled tissues were used for parallel extraction of nucleic acids by the CTAB method and RNeasy and/or DNeasy kits, respectively. For *R. solanacearum*-inoculated geranium, however, a 200-mg stem spanning the crown was collected from each plant and divided equally for DNA extraction by the CTAB method and the DNeasy kit, respectively.

Total nucleic acids were extracted with a modified CTAB method as follows. The tissues (100 mg) were added to a 2.0-ml screw cap microcentrifuge tube (Fisher Scientific, USA) containing two 1/4 in. stainless steel slingshot beads (Crosman Corp., USA) and 1 ml of CTAB buffer (2% CTAB, 2% PVP-40, 100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, and 0.2% 2-mercaptoethanol). The capped tube was first cooled at −20 °C for 5–15 min until the contents of the tubes were partially frozen and then processed at a speed setting of 4.5 for 60 s in a FastPrep® Instrument (Savant Instruments, Inc., Holbrook, NY, USA). The homogenate was incubated at 65 °C for 15 min and centrifuged at 10,000*g* for 5 min. The supernatant (650 μl) was transferred to a 1.5-ml microcentrifuge tube and mixed with an equal volume of chloroform/isoamyl alcohol (24:1) before centrifugation at 15,000*g* for 10 min. The supernatant (500 μl) was transferred to a 1.5-ml microcentrifuge tube containing 350 μl of isopropanol, and the mixture was centrifuged at 15,000*g* for 10 min. The pellet was washed with 70% ethanol by centrifugation at 15,000*g* for 5 min, air-dried and dissolved in 100 μl of 20 mM Tris–HCl, pH 8.0. The pooled plant tissue (0.5 g) was ground in liquid nitrogen into fine powder. One hundred milligrams of the tissue powder were used to obtain total RNA or DNA with the RNeasy kit or DNeasy kit according to the manufacturer's instructions, and the final elute was adjusted to 100 μl with sterile water. The extracts were stored at −20 °C for short term (≤2 months) or −80 °C for long term (≥3 months). Aliquots of an extract were stored if multiple tests of a pathogen or monoplex detection of multiple pathogens were planned. RNase inhibitor (0.8 U/μl) was added to an extract to prevent RNA degradation if target pathogen(s) contain RNA genome(s).

The quality of the extracts was measured using a NanoDrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and visualized by electrophoresis through 1.5% agarose gels.

2.3. Primers and probe

Primer pairs and probe used in this study are listed in Table 2. Primers for RT-PCR amplification of *American plum line pattern virus* (APLPV), *Apple dimple fruit viroid* (ADFVd), Apple fruit crinkle viroid (AFCVd), *Apple scar skin viroid* (ASSVd), *Cherry virus A* (CVA), *Fiji disease virus* (FDV), *Pear blister canker viroid* (PBCVd), *Prunus dwarf virus* (PDV), *Peach latent mosaic viroid* (PLMVd), *Ryegrass mosaic virus* (RGMV), *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato virus G* (SPVG) were designed based on alignment of sequences of each pathogen available in the GenBank. These primers were selected to bind to conserved regions of target pathogens if multiple sequences of different isolates were available. The primers for Lolium Latent virus (LoLV) was designed based on its available sequence (Maroon-Lango et al., 2005).

2.4. RT-PCR, PCR and real-time PCR

RT-PCR for detection of RNA viruses and viroids were carried out as described by Li and Mock (2005) using the OneStep RT-PCR Kit (Qiagen). PCR for detection of DNA viruses was performed according to Li et al. (2004) using *Taq* DNA polymerase (Invitrogen Carlsbad, CA, USA). Both RT-PCR and PCR programs varied in the annealing temperature and extension time according to primer combination and length of the DNA fragment, respectively. The general thermal cycling conditions for RT-PCR were 1 cycle of 95 °C for 15 min, 50 °C for 45 min and 95 °C for 10 min, 35 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min and one final extension at 72 °C for 8 min. The general thermal cycling conditions for PCR were 35 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min and one final extension at 72 °C for 8 min.

Nested PCR for detection of phytoplasmas were performed using HotStar *Taq* DNA Polymerase (Qiagen) as described by Lee et al. (1993) with modification. The initial PCR was done by adding 1 μl of the extract to 29 μl of the PCR master mix containing a final concentration of 1× *Taq* buffer, 0.2 mM dNTP, 0.4 μM each of the 16SrX group-specific primers R16mF2/R1, and 1 unit of *Taq* DNA polymerase. The nested amplification was done with the primers R16mF2N/R2 using 1 μl of the diluted amplicon (1:50) obtained from the initial PCR. Amplification was done using 1 cycle of 10 min at 95 °C, 35 cycles of 1 min at 94 °C, 2 min at 50 °C (60 °C for the nested primers) and 1 min at 72 °C, and a final extension of 10 min at 72 °C.

PCR for detection of *R. solanacearum* was performed by adding 1 μl of the extract to 24 μl of the PCR master mix (Qiagen) containing a final concentration of 1× *Taq* buffer, 1× Q buffer, 0.125 mM dNTP, 5 pmol each of *R. solanacearum*-specific primers 759 and 760 (Opina et al., 1997), and 1 unit of *Taq* DNA polymerase (Qiagen). Amplification was done using 1 cycle of 4 min at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 66 °C and 1 min at 72 °C, and a final extension of 10 min at 72 °C. *R. solanacearum* genomic DNA (10 ng) purified from strain K60 was used as a positive control and water as a negative control.

*Taq*Man real-time PCR assay for detection of the Las B232 was carried out as described by Li et al. (2006).

2.5. Analysis of PCR amplified products

Aliquots (8 μl) of PCR amplified DNA were analyzed by electrophoresis at 80 V for 1 h through ethidium bromide-stained agarose gels (1.2–1.5%) in 1× TAE buffer (40 mM Tris–acetate, 10 mM EDTA, pH 8.0). The size of amplified PCR products was determined using 1 kb DNA ladder or 1 kb plus DNA ladder (Invitrogen). Separated fragments were visualized using a FluroChem HD2 Imaging System (Alpha Innotech Co., San Leandro, CA, USA).

3. Results

3.1. Homogenization of plant tissues

With the FastPrep® Instrument, plant tissues including dormant budwood of fruit trees were effectively homogenized, and 12–24 samples could be homogenized simultaneously in less than 2 min. This step avoids the intensive labor involved and possible contamination that can occur when grinding samples in liquid nitrogen with mortars and pestles, an important step in the protocol of the RNeasy kit. Therefore, 48 samples can be easily processed by a person in a single day. Temperature during homogenization of the samples had an affect on the quality of RNA, but not DNA. Disruption of plant tissues at room temperature did not affect the PCR amplification of *Sweet potato leaf curl virus* (SPLCV), a DNA virus (Fig. 1A, lanes 3–5), but reduced the efficiency of RT-PCR amplification of SPVG, an RNA virus, to below detectable levels (Fig. 1A, lane 9). Therefore, pre-cooling tubes before sample disruption was used throughout the

Table 2
List of primers and probe used in this study.

Primer	Sequence (5′–3′)	Target	Product size	Reference
CLS6860	TTCATGGAAAGACAGGGGCAA	ALCSV	677 bp	Spiegel et al., 2006
CLS7536	AAGTCTACAGGCTATTTATTATAAGTCTAA			
ASGV1	GCCACTTCTAGGCAGAACTCTTTGAA	ASGV	273 bp	Menzel et al., 2002
ASGV2	CATCCCTTCTCCCTTGAGCA			
ASPV1	ATGTCTGGAACCTCATGCTGCAA	ASPV	370 bp	Menzel et al., 2002
ASPV2	TTGGGATCAACTTTACTAAAAAGCAT			
BR1	ACGTTAGCTTGCAGTCCCAC	BRV	426 bp	Jones and McGavin, 2002
BR4	CCAATCGTTCGAGGTGGGGCTCC			
APLPV1	GACGAGGCTGATGAAGGGAA	APLPV	501 bp	This study
APLPV2	ACCCACAGAAGGACCTACCA			
CGRMV1	CCTCATTCACATAGCTTAGGTTT	CGRMV, CNRMV	958, 959 bp	Li and Mock, 2005
CGRMV2	ACTTTAGCTTCGCCCCGTG			
CVA-Li1	GTGGCATTCAACTAGCACCTAT	CVA	873 bp	This study
CVA-Li2	TCAGCTGCCTCAGCTTGGC			
cPPV(3′)	GTCTCTTGCACAAGAACTATAACC			
LCUW7090	GGTTGTCCTCGGTTGATTAC	LCV-1	300 bp	Bajet et al., 2008
LCUWc7389	GGCTTGGTTCCATACATCTC			
hPPV(3′)	GTAGTGGTCTCGGTATCTATCATA	PPV	243 bp	Wetzel et al., 1991
PDV1	GGAAAACCTACTGCCCGTTC	PDV	540 bp	This study
PDV4	ATCGAGTGTTGGAGGTACTGAGT			
PNRSV F	CTTGAAGGACCAACCGAG	PNRSV	351 bp	Mekuria et al., 2003
PNRSV R	ATCTGCTAACGCAGGTAAG			
Shu-F	TACGGTAAGTGCCCAACTCC	BYDVs	830 bp	Malmstrom and Shu, 2004
Yan-R	TGTTGAGGAGTCTACCTATTTG			
S2a-F	TCACCTTCGGGCCGTCTCTATCAG	CYDV-RPV	372 bp	Malmstrom and Shu, 2004
Yan-R	TGTTGAGGAGTCTACCTATTTG			
LoLV1	AATGACAGAGTTCCCGAGCT	LoLV	561 bp	This study
LoLV2	GAAGTGGATGTGCTCAGTCG			
RgMV1	AAGTGGTRCACGAAGCTAACG	RgMV	955 bp	This study
RgMV2	CATATTCGCACCCGAGAGTG			
FDVC9	ACCATCTTCAGGTAAGCTTG	FDV	458 bp	This study
FDVC10	CAGCTGAATCGAAAGGTAACG			
SCMVF4	GTTTTYCACCAAGCTGGAACAGTC	SCMV	878 bp	Alegria et al., 2003
SCMVR3	AGCTGTGTGTCTCTCTGTATTCTTC			
SrMVF3	AAGCAACAGCACAAGCAC	SrMV	907 bp	Alegria et al., 2003
SrMVR3	TGACTCTCACCGACATTCC			
ST2	GAATTGGCGTACAAGTG	SCSMV	400 bp	Rott (personal communication)
ST5	ACTAAGCGGTCAGGCAAC			
SCYLV1	GATTGTGCGATCCGATTCG	SCYLV	1154 bp	This study
SCYLV2	GCCGAGTCTGTCTACGACG			
SPFMV3	AAYGGAYTRATGGTWTGGTGCAT	SPFMV, SPVG, SPGY	458–467 bp	This study
SPFMV4	TGCACACCCCTCATTCCYAAGAG			
GVB3F	GACGATGAATCCCTGAGAACC	GVBaV	527 bp	Jones and McGavin, 2001
GVB3R	CAGAAGTTAAGCCAGCGAACC			
SPG1	CCCCKGTGCGWRAATCCAT	SPLCV	912 bp	Li et al., 2004
SPG2	ATCCVAAYWTYCAGGGAGCTAA			
ADFVd3	GAGGAAAACTCCGTGTGGTTC	ADFVd	274 bp	This study
ADFVd4	AGCTCCACTCCCTGCCAGACC			
AFCVD1a	TGGGCTCCAACTAGTGGTTCC	AFCVd	353 bp	This study
AFCVD1b	GTCTCCTAGGTATCCACGACCG			
ASSVd1a	ACACCGTGCGGTTCCTGTG	ASSVd	242–249 bp	This study
ASSVd2b	GTCGAGCGGACTCCGGGT			
VP19	GCCCCGGGGCTCCTTTCTCAGGTAAG	HSVd	300 bp	Astruc et al., 1996
VP20	CCCGGGGCAACTCTTCTCAGAATCC			
PBCVd1	GCTCCCCTGACCTGCGTTCC	PBCVd	272 bp	This study
PBCVd2	GGTCCGCGGTAAACTTCCACC			
PLMVd1	ACCTCTCAGCCCYTCCACCTT	PLMVd	248–250 bp	This study
PLMVd2b	AGAGACTCATCAGTGYGCTWAGC			
R16mF2	CATGCAAGTCGAACGGA	Phytoplasma	1.4 kb	Lee et al., 1993
R16mR1	CTTAACCCCAATCATCGAC			
R16F2N	GAAACGACTGCTAAGACTGG		1.2 kb	
R16R2	TGACGGGCGGTGTGTACAAACCCCG			
759	GTCGCCGTCAACTCACTTTCC	*Ralstonia solanacearum*	281 bp	Opina et al., 1997
760	GTCGCCGTCAGCAATGCGGAATCG			
HLBas	TCGAGCGCGTATGCAATACG	*Ca.* Liberibacter asiaticus	75 bp	Li et al., 2006
HLBr	GCGTTATCCCGTAGAAAAAGGTAG			
HLBp	AGACGGGTGAGTAACGCG			
COXf	GTATGCCACGTCGCATTCCAGA	Citrus	68 bp	
COXr	GCCAAAACTGCTAAGGGCATTC			
COXp	ATCCAGATGCTTACGCTGG			

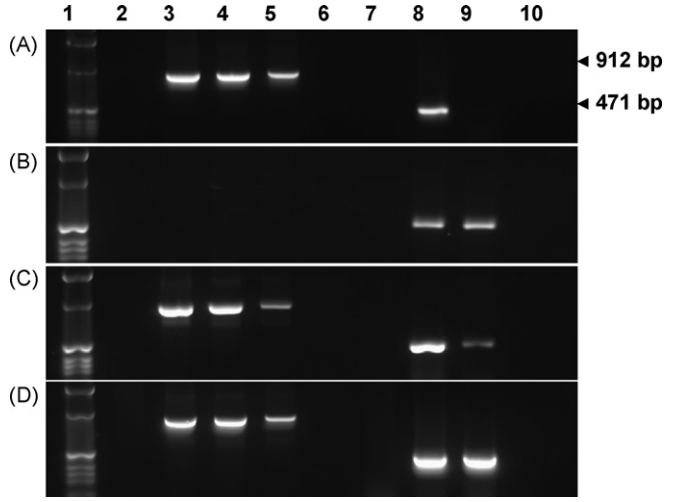

Fig. 1. Evaluation of the CTAB method for sample preparation for the PCR-based detection of *Sweet potato leaf curl virus* (SPLCV, lanes 3–6), *Sweet potato feathery mottle virus* (SPFMV, lane 8) and *Sweet potato virus G* (SPVG, lane 9) in sweet potato plants. The amplifications were done using the extracts obtained: A, without precooling; B, before isopropanol precipitation with precooling; C, after isopropanol precipitation with precooling; D, by the Qiagen commercial kits as control. Lanes: 1, 1 kb DNA ladder; 2 and 7, healthy control; 6 and 10, water control.

study. To reduce expense, steel beads were used to replace 1/4 in. ceramic spheres (Qbiogene, Morgan Irvine, CA, USA), and comparative study showed that the steel beads did not have an adverse effect on the extracts (data not shown).

3.2. Quantity and quality of the nucleic acids

The effect of chloroform/isoamyl alcohol extraction and isopropanol precipitation in the CTAB method was evaluated by PCR/RT-PCR using extracts collected before each step, respectively. None of SPLCV, SPFMV and SPVG could be detected in extracts collected prior to the chloroform/isoamyl alcohol step (data not shown). SPLCV was not amplified from extracts obtained before isopropanol precipitation (Fig. 1B, lanes 3–5), while SPFMV and SPVG could be amplified from extracts obtained either before or after precipitation with 70% isopropanol (Fig. 1B and C, lanes 8–9). This indicates that extraction with chloroform/isoamyl alcohol removed most, but not all, PCR inhibitors.

The average yields of total nucleic acids from 100 mg of plant materials using the CTAB method ranged from 25.7 μg (*Ribes* spp.) to 73.2 μg (*Prunus* spp.), much higher than those obtained from either the Qiagen DNeasy kit or RNeasy kit since the extracts contained both DNA and RNA (Table 3). The average ratios of spectrophotometric absorbance A260/A280 were 2.03–2.11 for the extracts prepared by the CTAB method, indicating the nucleic acids obtained were at least as pure as most of the extracts prepared by the Qiagen kits and contained both DNA and RNA (Table 3). The quality of the CTAB extracts was further confirmed by the presence

Table 3
The average yields and A260/280 ratios of total nucleic acids prepared by the CTAB method, the Qiagen DNeasy and RNeasy Plant Mini Kits.

	Yield[a] (μg/0.1 g)			A260/A280[a]		
Plant type	CTAB	DNeasy	RNeasy	CTAB	DNeasy	RNeasy
Ipomoea spp.	69.2	1.0	19.0	2.11	1.74	2.10
Prunus spp.	73.2	1.6	8.0	2.03	1.91	2.07
Malus spp.	59.5	1.5	1.5	2.11	1.73	1.89
Ribes spp.	25.7	1.0	2.2	2.08	1.71	1.57

[a] Average of at least four extracts from different plants.

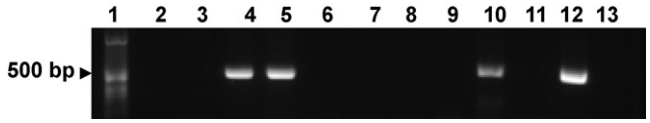

Fig. 2. Comparison of the CTAB and the commercial extraction methods for the PCR detection of *Gooseberry vein banding associated virus* (GVBaV, lanes 2–6) and RT-PCR detection of *Blackcurrant reversion virus* (BRV, lanes 7–13) in *Ribes* spp. Lanes: 1, 1 kb DNA ladder; 2–3, healthy blackcurrant plant; 4–5, a gooseberry plant infected with GVBaV; 6, water control; 7–8, a healthy blackcurrant plant; 9–12, blackcurrant plants infected with BRV; 13, water control. Extracts used as PCR templates were prepared by the CTAB method (lanes 3, 5, 8, 10 and 12), the Qiagen DNeasy kit (lanes 2 and 4) and the Qiagen RNeasy kit (7, 9 and 11), respectively. The tests were performed using pooled tissues of senescing leaves, petioles and bark.

of intact plant rRNA bands on the agarose gels after electrophoresis (data not shown). The total RNA obtained with the RNeasy kit from *Ribes* spp. had lower 260/A280 ratios (Table 3), indicating the presence of proteins, and was not suitable for the RT-PCR amplification of *Blackcurrant reversion virus* (BRV) from infected *Ribes* spp. (Fig. 2, lanes 9 and 11). The efficient PCR amplification of a range of targets confirmed that the CTAB method yielded total nucleic acids with high quantity and quality (Table 1).

3.3. RT-PCR for detection of RNA viruses and viroids

Extracts prepared by the CTAB method and RNeasy kit were compared by RT-PCR using the following combinations of pathogens and infected plants (Table 1): sweet potato plants infected with *Sweet potato chlorotic stunt virus* (SPCSV), SPFMV (Fig. 1, lane 8), SPGV (Fig. 1, lane 9) and *Sweet potato virus Y* (SPVY); blackcurrant plants infected with *Blackcurrant reversion virus* (BRV) (Fig. 2, lanes 9–12); apple trees infected with *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV), ADFVd, ACFVd and ASSVd; a pear tree infected with PBCVd; stone fruit trees infected with APLPV, *Cherry green ring mottle virus* (CGRMV), *Cherry necrotic rusty mottle virus* (CNRMV) isolates, *Cherry virus A* (CVA), *Little cherry virus 1* (LCV-1), *Plum pox virus* (PPV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV), *Hop stunt viroid* (HSVd) and PLMVd; ryegrass plants infected with LoLV and RGMV; sugarcane plants infected with SCMV, SCSMV, SCYLV and SrMV; The expected products were amplified from most targets except BRV using the nucleic acids prepared by both methods. BRV could only be detected from the extracts prepared with the CTAB method (Fig. 2, lanes 10 and 12).

The CTAB method was used successfully to prepare suitable nucleic acids from actively growing cherries and sweet potatoes. These plants typically have a high content of polysaccharides that frequently clog the QIAshredder Mini Spin Columns and make the extraction difficult (data not shown). The CTAB method was also used to prepare samples from leaves, petioles, bark and dormant budwood of infected plants for PCR-based detection of a range of plant RNA viruses including *Barley yellow dwarf virus* (BYDV)-MAV, BYDV-PAV and *Cereal yellow dwarf virus*-RPV infecting barley, and FDV infecting sugarcane (Table 1).

3.4. PCR for detection of DNA viruses

Extracts prepared by the CTAB method and DNeasy kit were compared by PCR using sweet potato (*Ipomoea batatas*) cultivars infected with SPLCV (Fig. 1, lanes 3–5), a gooseberry plant with *Gooseberry vein banding associated virus* (GVBaV) (Fig. 2, lanes 4 and 5). Results indicated that DNA fragments of SPLCV and GVBaV were amplified from the nucleic acids prepared by both methods from infected plants. To confirm that good-quality DNA was obtained,

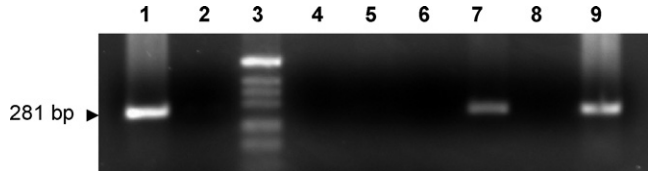

Fig. 3. Comparison of the CTAB and Qiagen DNeasy Plant Mini Kit extraction methods by the PCR detection of *Ralstonia solanacearum* in inoculated geranium plants. Lanes: 1, positive control (*R. solanacearum* genomic DNA); 2, water control; 3, 1 kb DNA ladder; 4, 6 and 8, geranium DNA extracted using the DNeasy kit from a healthy plant (DI = 0), a plant with ≤25% leaves wilted (DI = 1), and a totally wilted plant (DI = 4), respectively; 5, 7 and 9, geranium DNA extracted using the CTAB method from DI = 0, 1 and 4 plants, respectively.

the CTAB method was further compared with plant DNAzol buffer method (Lotrakul and Valverde, 1999) for sample preparation using sweet potato and two graft inoculated indicator plants, *I. nil* and *I. setosa*. The expected PCR bands with similar intensity were detected in extracts obtained by both methods from infected plants (data not shown). The data indicate that the CTAB method is suitable to extract DNA for the PCR detection of SPLCV in sweet potato, a known recalcitrant host plant.

3.5. PCR-based techniques for detection of phytoplasma and bacterial pathogens

The CTAB method was compared with the DNeasy kit for sample preparation from infected plants for PCR-based detection of phytoplasmas and bacteria. The expected size of PCR product was amplified from extracts prepared by both methods from an apple tree infected with apple proliferation and peach trees infected with peach yellow leaf roll (Table 1). A more intense band was visualized in the agarose gel from the nested PCR using extracts prepared by the CTAB method (data not shown).

Total nucleic acids extracted with the CTAB method from inoculated geranium plants showing wilt symptoms caused by *R. solanacearum* yielded visible PCR bands with the expected DNA size, while no PCR product was observed when total DNA was extracted from the same diseased plants using the DNeasy kit (Fig. 3, lanes 4, 6 and 8). The results suggest that the CTAB method is much more effective in extracting bacterial DNA from geranium plants and/or removing PCR inhibitors.

Extracts obtained from 8 Las B232-infected and 4 healthy rough lemon trees were analyzed by the *Taq*Man PCR using HLBaspr (specific primers and probe for the pathogen) and COXfpr (specific primers and probe for plant mitochondrial cytochrome oxidase).

Table 4
Comparison of sample preparation by the CTAB extraction method and Qiagen DNeasy Plant Mini Kit in TaqMan PCR for detection of *Ca.* Liberibacter asiaticus.

Sample no.	Ct value of host DNA		Ct value of Las B232* DNA	
	DNeasy	CTAB	DNeasy	CTAB
B232#1	20.7	19.9	20.9	20.4
B232#2	19.4	20.0	19.8	20.2
B232#3	20.2	20.0	20.1	20.3
B232#4	20.3	19.7	20.4	20.3
B232#5	19.5	19.8	19.7	19.9
B232#6	19.9	19.4	20.1	19.6
B232#7	19.8	19.3	19.9	19.6
B232#8	19.9	20.6	20.1	20.9
HC**#1	21.7	20.4	0.0***	0.0
HC#2	22.6	22.2	0.0	0.0
HC#3	21.1	20.1	0.0	0.0
HC#4	22.9	19.6	0.0	0.0
Water	0.0	0.0	0.0	0.0

Las B232 = isolate B232 of *Candidatus* Liberibacter asiaticus; **HC = Healthy control; ***0.0 = did not cross the threshold.

The Ct values of samples prepared by the CTAB method were 19.3–22.2 for COXfpr and 19.6–20.9 for HLBaspr, respectively, comparable with those prepared by the DNeasy kit (Table 4). Our results, consistent with those of Li et al. (2006), indicate that the quality of DNA extracted by the CTAB method from citrus plants was sufficient for the detection of *Candidatus* Liberibacter.

4. Discussion

Plant quarantine and certification programs are typically required to target multiple pathogens (often viruses and virus-like) in either a single crop or many different crops. Six different protocols including RNeasy kit were initially used in our laboratories for extraction of either total RNA or DNA to be used in PCR-based detection of a diverse group of pathogens including RNA and DNA viruses, viroids and phytoplasmas from a range of host plants including sweet potato, small fruits and fruit trees. The use of multiple extraction protocols is laborious, time-consuming and costly; therefore, development of a 'universal' method for extraction of total nucleic acids from different host plants would be very useful. Such a protocol must be able to obtain extracts containing both DNA and RNA, and be simple, fast and cost-effective for processing large-scale samples from many host plants.

The data presented in this paper demonstrate that the modified CTAB method is suitable to prepare extracts for PCR-based detection of all plant pathogens tested, a diverse group including 26 RNA viruses, two DNA viruses, six viroids, two phytoplasmas and two bacteria from the extracts obtained from different types of plants. CTAB-based methods have been previously reported for extraction of high-quality nucleic acids from plants, especially from plants with high levels of polyphenols and polysaccharides (Allen et al., 2006; Bekesiova et al., 1999; Chang et al., 1993; Flagel et al., 2005; Jaakola et al., 2001; Kim and Hamada, 2005; Puchooa, 2004; Wang et al., 2005, 2008). However, most described applications as well as the manufacturer's protocols for the Qiagen commercial kits require grinding plant tissue with mortars and pestles in liquid nitrogen, a process that is laborious and can lead to contamination between samples caused by splashing and reuse of labware. This step is not suitable for handling large numbers of samples. The FastPrep® Instrument is an apparatus which provides high-throughput processing for effective disruption of plant tissues, even woody materials such as dormant buds and bark of fruit trees, making it the choice for sample homogenization of various plant species. The process is simple and fast, allowing a large number of samples to be processed each day. Cross contamination between samples at the homogenization stage was prevented by processing each sample in a sealed tube. Temperature during sample disruption affected the quality of total RNA, and therefore precooling before sample disruption can minimize RNA degradation during homogenization. Extracts stored at −80 °C for 6 months resulted in no loss of amplification (data not shown). The material cost of the CTAB method was reasonable, approximately 33% and 20% of the DNeasy and RNeasy kits, respectively. The FastPrep® Instrument is relatively expensive, but the 'universal' property of the extracts and labor involved with the CTAB method can easily justify the initial cost.

After sample disruption, nucleic acids are extracted with chloroform/isoamyl alcohol and precipitated with isopropanol. The chloroform/isoamyl alcohol extraction removed most proteins and PCR inhibitors from sweet potato leaves, as indicated by the amplification of SPFMV and SPVG in the extracts without isopropanol precipitation, and was an indispensable step of the method. The failure to amplify SPLCV fragment from the extracts without isopropanol precipitation suggests that *Taq* DNA polymerase was more sensitive to the presence of PCR inhibitors in these extracts than the Enzyme Mix in the OneStep RT-PCR kit (Fig. 1B, lanes 3–5).

Isopropanol precipitation reduced the extract volume and yielded colorless extracts lacking inhibitors for the *Taq* DNA polymerase (Fig. 1C, lanes 3–5).

The modified CTAB method was compared with commonly used extraction kits such as RNeasy and DNeasy kits for extraction of nucleic acids from plants for PCR-based detection of different pathogens. Our comparative tests showed that the extracts (either total RNA or DNA) prepared by the DNeasy and RNeasy kits were suitable for the PCR/RT-PCR amplifications of most of our target pathogens (Tables 1 and 4). However, it was noted that BRV, a RNA virus with low virus titer in infected plants (Jones and McGavin, 2002), was not detected in extracts prepared by the RNeasy kit from senescing leaves, petioles and bark of infected plants (Fig. 2, lanes 9 and 11). Similarly, *R. solanacearum*, a bacterial pathogen, could not be detected in extracts prepared by the DNeasy kit from infected and symptomatic geranium plants (Fig. 3, lanes 6 and 8). The successful amplification of these pathogens could be the result of the high-yield extracts with low amounts of PCR inhibitors obtained by the CTAB method. Our results suggest that the CTAB method is efficient in removing RNA inhibitors present in these plants. We also found that extracts prepared by the RNeasy kit were suitable for the PCR detection of SPLCV, a DNA virus, indicating that the small viral DNA was not excluded by the method (data not shown). The extracts prepared by the CTAB method from these samples, however, allowed PCR amplifications of two DNA viruses (SPLCV and GVBaV) tested. High-yield and good-quality extracts suitable for use in PCR-based techniques were also obtained by the CTAB method from different tissues including bark and dormant budwood of fruit trees and *Ribes* spp. (Table 1; Fig. 2, lanes 10 and 12), allowing the routine detection of different viruses in infected plants throughout the year. These data indicated the quality of extracts prepared with the CTAB method was comparable, even better in some cases, than that extracted by the Qiagen commercial kits.

The CTAB method described in this paper has already been adopted for routine use in our laboratories for detection and PCR cloning of different pathogens in various host plants. Our protocol is preferable because of its universality, which is not only useful for monoplex PCR detection of different pathogens, but also the basis for future development of multiplex PCR for detection of different pathogens in single nucleic acid extracts of plant samples in a wide range of plant species.

Disclaimer

Information about commercial reagents and equipment is provided solely to assist the identification of suitable sources of such items. No specific endorsement or approval is intended and equivalents from other manufacturers may be equally suited.

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